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Purification and properties of apple russet ring virus.

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PURIFICATION AND PROPERTIES OF APPLE RUSSET RING VIRUS

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PURIFICATION AND PROPERTIES OF APPLE RUSSET RING VIRUS

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by

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CONTENTS

INTRODUCTION

Russet ring virus (RRV) of apple was found to infect McIntosh apples in Massachusetts (Agrios, 1965). RRV causes minute whitish-yellow flecks on leaves, small depressions and russet ring patterns on the skin of McIntosh apple fruit (Agrios, 1965). Similar fruit and foliage symptoms have been observed on several varieties in the state of Washington (Reeves and Cheney, 1960), where the virus causes losses equal to 11% of the yield (Welsh and Keane, 1957). To date, no chemical or physical properties of the virus have been studied. Although several cases in which viruses cause russet ring symptoms in apple have been reported in New York state (Palmiter, 1963), in Washington state (Reeves and Cheney, 1960) and in British Columbia (Welsh and Keane, 1957), no definite conclusions have been reached as to the identity of these viruses.

Since RRV disease of apple was first reported on the fruit of a small number of McIntosh apple trees in two widely separated orchards in Massachusetts (Agrios, 1965), no other literature on this virus disease has appeared. However, the virus has been transmitted mechanically from apple leaves and petals to the herbaceous hosts Chenopodium

amaranticolor and C. quinoa (Agrios and Chandrasekhar, 1969, unpublished data).

For the research described here, the virus was obtained and retransmitted from C. amaranticolor to C. amaranticolor and to bean plants (Phaseolus vulgaris). The general properties of the virus were determined in both crude juice and in sap partially clarified with magnesium bentonite. For electron microscopy, preparations were made both from purified virus and from crude leaf extracts.

It was the purpose of this research to purify the virus, and study some of its properties. This information should be helpful in establishing the identity of the virus, and in further research on the nature and control of this virus.

LITERATURE REVIEW

An apple fruit disorder resembling the russet ring disease was first shown to be graft-transmissible by Welsh and Keane (1957). Reeves and Cheney (1960) used the name "Russet Ring" to describe a similar disorder, although russet ring symptoms on fruit had been observed on orchard trees in the state of Washington as early as 1935 (Starcher, 1960).

Palmiter (1963) found open or solid russet rings on the fruit of Lord Lambourne and Golden Delicious apple varieties in New York state, and considered these symptoms to be caused by green mottle virus or a mixture of viruses. McIntosh, however, was one of the varieties from which the green mottle disease could be transmitted, but on which it did not produce any symptoms.

Palmiter in 1969 reported another mechanically transmissible virus, which he called "McIntosh Depression Virus". This virus caused foliage mosaic and a serious fruit deformity in the form of irregular depressions, however, it did not produce russet rings on McIntosh apples in New York state. Definite russet rings were induced by this virus on fruit of Malus platycarpa only.

The virus studied here typically causes leaf flecking and russet rings on McIntosh apples in Massachusetts

orchards (Agrios, 1965). The relationship, if any, of this virus to those reported by other workers is presently unknown.

Although more than 24 viruses (McCrum et al., 1960; Cheney et al., 1970; Welsh and May, 1970; Palmiter, 1970) cause symptoms on apple, only a few of them, namely, Tulare apple mosaic virus (Mink and Bancroft, 1962), apple stem pitting virus (Apple latent virus-2, Kirkpatrick et al., 1965), apple chlorotic leafspot virus (Saksena and Mink, 1969) and filamentous apple virus isolates C-431 and E-36 (de Sequeira and Lister, 1969) have been mechanically transmitted and purified. All these viruses have been mechanically transmitted from apple to the herbaceous hosts Chenopodium amaranticolor and C. quinoa; in addition, Tulare apple mosaic virus has been transmitted to Nicotiana tabacum L. var. Connecticut Havana 423 (Mink and Bancroft, 1962); filamentous apple virus isolates C-431 and E-36 have been transmitted to Nicotiana glutinosa and Phaseolus vulgaris (de Sequeira and Lister, 1969); and apple chlorotic leafspot virus has been transmitted to Phaseolus vulgaris L. varieties Kinghorn, Bountiful and 'D-9' (Saksena and Mink, 1969).

For clarification of virus extracts, the use of bentonite by Cadman (1963) proved very successful, even

though absolute standardization could not be achieved because the amount of bentonite required depended on the age and the condition of plants (Lister et al., 1965). Previous experiments suggested that excess bentonite could reduce loss of virus during repeated cycles of ultracentrifugation (Lister et al., 1965).

Along with bentonite clarification, adjustment of pH and resuspension of the precipitate by acidification of, or by adding polyethylene glycol to, the partially clarified extracts, was used by de Sequeira and Lister (1969) for the purification of apple virus isolates C-431 and E-36. Clarification by heat denaturation and n-butanol precipitation of protein with differential centrifugation was used by Kirkpatrick et al.(1965).

Magnesium bentonite was first tested as a clarification agent by Dunn and Hitchborn (1965). Saksena and Mink (1969) applied it to the purification of apple chlorotic leafspot virus. Magnesium bentonite clarification yields a highly infectious virus preparation containing little detectable host contaminants.

Purification has been accomplished by using C. amaranticolor, C. quinoa and Nicotiana tabacum as virus source plants (Fulton, 1965). After Hollings (1956) pointed out the suitability of C. amaranticolor as a

differential and assay plant for many viruses, C. quinoa has been used intensively for viruses latent in apple. It is apparently the best of the few herbaceous hosts to which these viruses have been transmitted (Pfaeltzer, 1962; Lister et al., 1965).

Various treatments aimed at increasing the susceptibility of plants to virus infection have been used, although critical data are not always available to determine whether such treatments were really effective. Darkening of plants before inoculation to increase susceptibility has been known for some time (Bawden, 1947) and has been used quite frequently.

Purification of plant viruses is generally carried out by using different buffer solutions. Thornberry (1935) first demonstrated that K_2HPO_4 and Na_2HPO_4 increased infection of tobacco mosaic virus on bean. Yarwood (1952) reported that a greater number of local lesions were produced when bean leaves were inoculated with virus obtained by grinding apple mosaic-infected apple leaves in phosphate buffer than when the virus was obtained by grinding apple mosaic-infected apple leaves in water. Abrasive carborundum alone was more effective than phosphate buffer, but together they were more effective than either was alone, and produced more local lesions than the sum of

the local lesions of the two applied separately (Yarwood, 1952).

Mink and Bancroft (1962) found that infectivity of apple mosaic virus could be retained for at least 23 hr at 25°C by macerating leaves in an aqueous solution of 0.01 M sodium diethyldithiocarbamate (DIECA) and 0.01 M cysteine hydrochloride. The method of preparation of this solution had a profound effect on its stabilizing ability. Simultaneous dissolution of a mixture of the reagent resulted in a loss of stabilizing properties. Greatest stabilization was obtained when 0.02 M cysteine hydrochloride was added to an equal volume of 0.02 M DIECA (Mink and Bancroft, 1962). A pale yellow color developed shortly after mixing and increased in intensity for about 2 hr, but the ability to stabilize was unaltered during this time. Apple stem pitting virus (or apple latent virus-2, Kirkpatrick and Lindner, 1964) was purified in neutral 0.01 M phosphate buffer containing 0.01 M cysteine-HCl and 3% nicotine sulfate. For the filamentous apple virus isolates C-431 and E-36, de Sequeira and Lister (1969) used 0.05 M phosphate buffer at pH 7.8, without any anti-oxidant, throughout the purification.

Different purification procedures have been applied to apple viruses. Tulare apple mosaic virus (Mink and

Bancroft, 1962) was extracted by grinding frozen infected tissue into a coarse powder at -22°C, mixing with the buffer solutions containing 0.02 M cysteine-HCl and 0.02 M DIECA and infiltrating *in vacuo* at room temperature for 1 hr. The tissue was softened, the excess buffer was poured out and 8.5% (v/v) n-butanol was added for clarification. The partially clarified sap was subjected to two cycles of differential centrifugation at 39,000 rpm in a Spinco model L No. 40 rotor for 1 hr. High-speed pellets were resuspended in 0.01 M acetate buffer pH 4.9 giving a water-clear solution that differed greatly from the control in ultraviolet absorption.

Apple chlorotic leafspot virus (Saksena and Mink, 1969) was purified by grinding 25g samples of *C. quinoa* tissue collected 7 to 15 days after inoculation and homogenized for 2 min in a Waring Blendor with 2 volumes of buffer solution consisting of freshly mixed 0.02 M DIECA and 0.02 M cysteine-HCl in equal volumes. The anti-oxidant and reducing agent were included to reduce the coloration in the final pellet. Mg-bentonite (1 ml/5g of tissue) was added, the mixture was stored at 4°C for 10 to 15 min and was then centrifuged for 15 min at 10,000 rpm in a Sorvall SS-1 centrifuge to remove the leaf material. The clear yellow supernatant was filtered through glass wool and centrifuged at 39,000 rpm in a Spinco

No. 40 rotor for 1.5 hr. The small, yellow translucent pellets were resuspended immediately in 0.01 M neutral phosphate buffer. After shaking for 5 to 10 min they were centrifuged again for 10 min at 10,000 rpm in a Spinco No. 40 rotor. The final, faint yellow supernatant was highly infectious.

The apple stem pitting virus (apple latent virus-2) purified by Kirkpatrick et al. (1965) was found to be stable at 60°C. Therefore, it was possible to purify the virus from heat-clarified homogenates of infected leaves of C. amaranticolor by differential centrifugation. Infected tissue was ground with 10 volumes of neutral 0.01 M phosphate buffer containing 0.01 M cysteine-HCl. The crushed material was pressed through one layer of cheese-cloth. The filtrate was heated at 60°C for 15 min with stirring. The preparation was centrifuged at 74,000g for 1 hr. The high-speed pellet was resuspended in 3 ml 0.01 M neutral phosphate, homogenized for 15 min, and the mixture was clarified at 6,000g for 10 min. The preparation was given a second high-low-speed centrifugation, and the pellet was suspended again in 3 ml of 0.01 M neutral phosphate buffer or 3 ml of 0.02 M phosphate buffer plus 0.15 M potassium chloride. The final pellet from virus infected tissue was a clear pale yellow gel.

The filamentous apple virus isolates C-431 and E-36 were purified by cycles of ultracentrifugation of doubly clarified extracts from systemically infected plants, followed by fractionation of the products by rate-zonal density gradient centrifugation (de Sequeira and Lister, 1969).

Purification of apple viruses is generally accomplished by density gradient centrifugation. The principles and procedures used were described by Brakke (1967). In density gradient centrifugation both rate-zonal and equilibrium density gradient centrifugation procedures have been employed. The former is depended on the size and shape of the particles, while the latter on the density of the particles. After both types of density gradient centrifugation, apple chlorotic leafspot virus showed a very clear zone 35 to 38 mm below the meniscus and this zone was highly infectious (Saksena and Mink, 1969).

RRV has been mechanically transmitted from young apple leaves and petals to C. amaranticolor and C. quinoa and from C. amaranticolor to several bean varieties, namely, Phaseolus vulgaris var. Bountiful stringless, Tenderbest, Kentucky Wonder and Pod Pencil Wax (Agrios and Chandrasekhar, unpublished data). No other information about the virus is available at present.

MATERIALS AND METHODS

THE VIRUS:

The RRV isolate used in these experiments was obtained from Chenopodium amaranticolor plants to which the virus had been previously transmitted mechanically (Agrios and Chandrasekhar, unpublished data) from McIntosh apple leaves and petals infected with RRV and collected at the University Orchard, Belchertown, Massachusetts.

THE HOSTS:

Russet ring virus was cultured in Chenopodium amaranticolor, C. quinoa and bean (Phaseolus vulgaris L.) varieties Bountiful stringless, Tenderbest, Kentucky Wonder and Pod Pencil Wax, grown in the greenhouse or in the growth chamber at a temperature of about 24°C. The light intensity maintained throughout experimentation varied between 500 and 800 ft-c with a 16 hr photoperiod. The greenhouse was supplied with auxiliary tungsten lighting during the winter months.

C. amaranticolor plants used as hosts for the virus were inoculated about 3 weeks after transplanting to 4-in. pots at which time they had 6 to 8 expanded leaves and were about 5 inches tall.

Plants were kept in darkness for 48 hr before they

were dusted with 600-mesh carborundum and inoculated. The plants were rinsed with water 20 to 30 min after inoculation to avoid injury by the chemical additives to the inoculum.

MECHANICAL INOCULATION:

Young leaves with systemic symptoms were harvested 3 to 4 weeks after inoculation. The leaves were chilled for 15 min before they were ground with mortar and pestle. Crude sap of the virus-infected plants was obtained by grinding the leaf material with the addition of 4 volumes distilled water, and subsequently pressing the homogenate through cheesecloth. For obtaining partially clarified sap, buffer solution (see below) and Mg-bentonite were added to the leaf material. The mixture was homogenized in a Virtis homogenizer for 2 min. The homogenate was centrifuged at 19,000 rpm for 15 min in a Sorvall RC2-B, SM-24 rotor. The clear, brownish-yellow supernatant was used as inoculum. Application of inoculum was made by gently rubbing the leaves with a cheesecloth dipped in the inoculum.

Buffer solution was made of neutral 0.01 M phosphate buffer containing 0.02 M sodium diethyldithiocarbamate and 0.02 M cysteine hydrochloride freshly mixed in equal

volumes. The volume in milliliters of buffer used was four times the weight in grams of leaf tissue used.

Magnesium bentonite suspension for homogenate clarification was prepared according to the method of Dunn and Hitchborn (1965). The suspension was made by suspending 100g bentonite in 2 liters of neutral 0.01 M phosphate buffer containing 0.01 M $MgSO_4$. After shaking for 2 hr, the mixture was centrifuged for 1 min at 1,500 rpm in the Sorvall RC2-B, SS-34 rotor. The pellet was discarded and the supernatant centrifuged for 15 min at 12,000 rpm. The pellet was resuspended in neutral 0.001 M phosphate buffer containing 0.001 M $MgSO_4$. After shaking again for 2 hr, the mixture was centrifuged for 1 min at 1,500 rpm. The pellet was then discarded and the supernatant centrifuged for 15 min at 12,000 rpm. The pellet was resuspended in 500 ml neutral 0.01 M phosphate buffer containing 0.001 M $MgSO_4$. The final suspension contained about 30% of the starting bentonite and the concentration was maintained at 50 mg/ml. The amount of Mg-bentonite used for clarification was 2 ml per 5g of tissue.

DETERMINATION OF GENERAL PROPERTIES:

For studying the aging of the virus <u>in vitro</u>, partially clarified sap containing the virus was stored at room

temperature (24°C). After 1/2, 1, 2, 4, 6, 8, 12, and 24 hr, the infectivity of the virus was assayed by inoculating C. amaranticolor plants with each of the stored saps. Four to 7 days later, the local lesions caused by the virus on each inoculated leaf were counted, and 3 to 4 weeks later, those of the inoculated plants that became systemically infected were also counted.

For studies of the dilution end point of the virus, both crude and partially clarified saps were used. The former was diluted with pure water and the latter with the phosphate buffer solution. The dilutions were 1:5, 1:10, $1:10^2$, $1:10^3$, $1:10^4$, $1:10^5$ and $1:10^6$. The inoculations were made by applying each dilution to leaves of C. amaranticolor plants. Both local lesions and systemic infections caused by each dilution were recorded.

The thermal inactivation point of the virus in sap was determined by heating crude sap and clarified sap in an electric water bath for exactly 10 min. The sap was put in small tubes of 1 cm diameter. The tubes were made to float in the water bath by surrounding them with a cork ring. The heated sap was cooled in an ice bath upon removal from the hot water bath. Inoculations were made immediately after cooling.

C. amaranticolor leaves showing systemic symptoms of

RRV were sealed in plastic bags and stored at -22°C in the freezer. After 1 day, 1 week, 2 weeks, 1 month, 2 months, 4 months and 6 months, the leaves were used for inoculation. Frozen tissue was ground with mortar and pestle in buffer solution containing 0.02 M DIECA and 0.02 M cysteine-HCl and then centrifuged at 19,000 rpm for 15 min in a Sorvall RC2-B, SM-24 rotor. The clear supernatant was used as inoculum.

The longevity of RRV in freeze-dried tissue was determined by freeze-drying C. amaranticolor leaves showing systemic symptoms in a Thermovac freeze-drier for about 3 days, grinding the freeze-dried leaves to a coarse powder and storing the powder in the freezer for periods of 1 day, 1 month, 6 months and 9 months, at which time the infectivity of the freeze-dried tissue was assayed. The freeze-dried powder was homogenized, clarified and inoculated as described above. Magnesium bentonite was added at the rate of 1 ml per 0.2g dry tissue powder.

PURIFICATION PROCEDURES:

The purification procedures used in this study were basically those used by Saksena and Mink (1969) with some minor modifications.

Twenty-five grams of RRV-infected C. amaranticolor

leaves showing systemic symptoms were harvested 3 to 4 weeks after inoculation. The young leaves were homogenized intermittently for about 2 min in a Waring Blendor with 4 volumes of freshly prepared buffer solutions.

Mg-bentonite was added (2 ml/5g of tissue) and the mixture was further homogenized for 1 min. The homogenate was stored for 15 min at 4°C, then centrifuged for 20 min at 10,000 rpm in a Sorvall RC2-B, SS-34 rotor to remove the coarse plant material. The clear brownish-red supernatant was filtered through glass wool and centrifuged for 60 min at 30,000 rpm in a No. 65 rotor of a Spinco L2-65B ultracentrifuge at 4°C. The small, yellowish-brown pellet was resuspended immediately in neutral 0.01 M phosphate buffer. After gentle shaking for 5 min, it was clarified by centrifugation at 10,000 rpm for 10 min in a Sorvall RC2-B, SM-24 rotor. The resulting faint yellow suspension was used as inoculum and was highly infective. The same suspension was also used for further purification of the virus by density gradient centrifugation.

DENSITY GRADIENT CENTRIFUGATION:

The density gradient centrifugation method used in this study was described by Brakke (1967). Rate-zonal density gradient tubes were made by layering 3 ml each of

the solutions containing 50, 100, 150, and 200g sucrose made up to one liter in 0.01 M neutral phosphate buffer. The procedures were carried out in a cold room, in which the temperature was kept at 4°C. All the tubes were stored at 4°C overnight before use.

The following day, 1 ml of purified virus suspension obtained from the last step of purification was layered on the rate-zonal sucrose density gradient tubes and centrifuged at 28,000 rpm for 4 hr in a Spinco L2-65B, SW 40 rotor.

After centrifugation, the content of the tubes was fractionated by puncturing a hole at the bottom of the gradient tube. Six drops (about 0.03 ml) of the content were collected in each test tube. Two ml neutral 0.01 M phosphate buffer was added to each of the test tubes; part of each fraction was inoculated on the leaves of C. amaranticolor to test for infectivity, while the remaining part of each fraction was examined under the ultraviolet light of a recording Baush and Lomb Spectronic 505 Spectrophotometer. The absorbance of each fraction at 260 and 280 mμ was recorded.

ELECTRON MICROSCOPY:

The fractions from the density gradient centrifuga-

tion which showed a good peak at 260 mu and also caused infection on the host plants were washed in neutral 0.01 M phosphate buffer and centrifuged at 30,000 rpm for 60 min in a No. 65 rotor of a Spinco L2-65B ultracentrifuge. The washed virus pellet was resuspended in 0.1 ml neutral 0.01 M phosphate buffer and was treated with 200 ug/ml ribonuclease Type 1A (Sigma Chemical Company). The mixture was incubated at 37°C for 1 hr to eliminate the remaining plant ribosomes and then was centrifuged at 30,000 rpm for 1 hr to precipitate the virus particles. The pellet was resuspended in neutral 0.01 M phosphate buffer and, after mixing with equal amount of 1% phosphotungstic acid, pH 7.05 for negative staining, was used for electron microscopy in a Phillips EM200 scope.

RRV preparations for electron microscopy were also made directly by sampling the systemically infected C. quinoa leaf tissue, homogenizing, and staining with potassium phosphotungstate at pH 7.0.

RESULTS

GENERAL PROPERTIES

Aging in vitro:

Russet ring virus survived for at least 4 hr in crude sap preparations stored at room temperature (24°C). At the end of that period the virus could still infect C. amaranticolor plants. In partially clarified sap, the virus lost its ability to infect C. amaranticolor after 6 hr storage at room temperature (Table 1, Fig. 1). This test was repeated 4 times with essentially identical results.

Dilution end point:

The virus in crude sap could still cause infection on C. amaranticolor plants after it had been diluted to $1:10^3$ while in partially clarified sap, a dilution of $1:10^4$ could still cause some local lesions on C. amaranticolor leaves (Table 2, Fig. 2). This experiment was repeated 7 times.

Thermal inactivation point:

Russet ring virus in crude sap and in partially clarified sap retained only a trace of its infectivity when heated at 48°C for 10 min (Tables 3,4,5 and Fig. 3).

Table 1. Longevity *in vitro* of RRV in crude sap and in clarified sap as assayed on *Chenopodium amaranticolor**

Time (hr)	No.of local lesions per leaf**		No. of plants systemically infected	
	Crude sap	Partially clarified sap	Crude sap	Partially clarified sap
1/2	27	35	10/10	10/10
1	14	16	7/10	10/10
2	7	12	4/10	6/10
4	3	3	1/10	4/10
6	0	2	0/10	1/10
8	0	0	0/10	0/10
12	0	0	0/10	0/10
24	0	0	0/10	0/10

*All data shown in this table are averages from 4 replicates.

**Average of the number of lesions of 6 inoculated leaves on each plant, from 4 replicates.

Table 2. Number of local lesions and systemic infections of serial dilutions of RRV in crude sap and in partially clarified sap inoculated on C. amaranticolor*

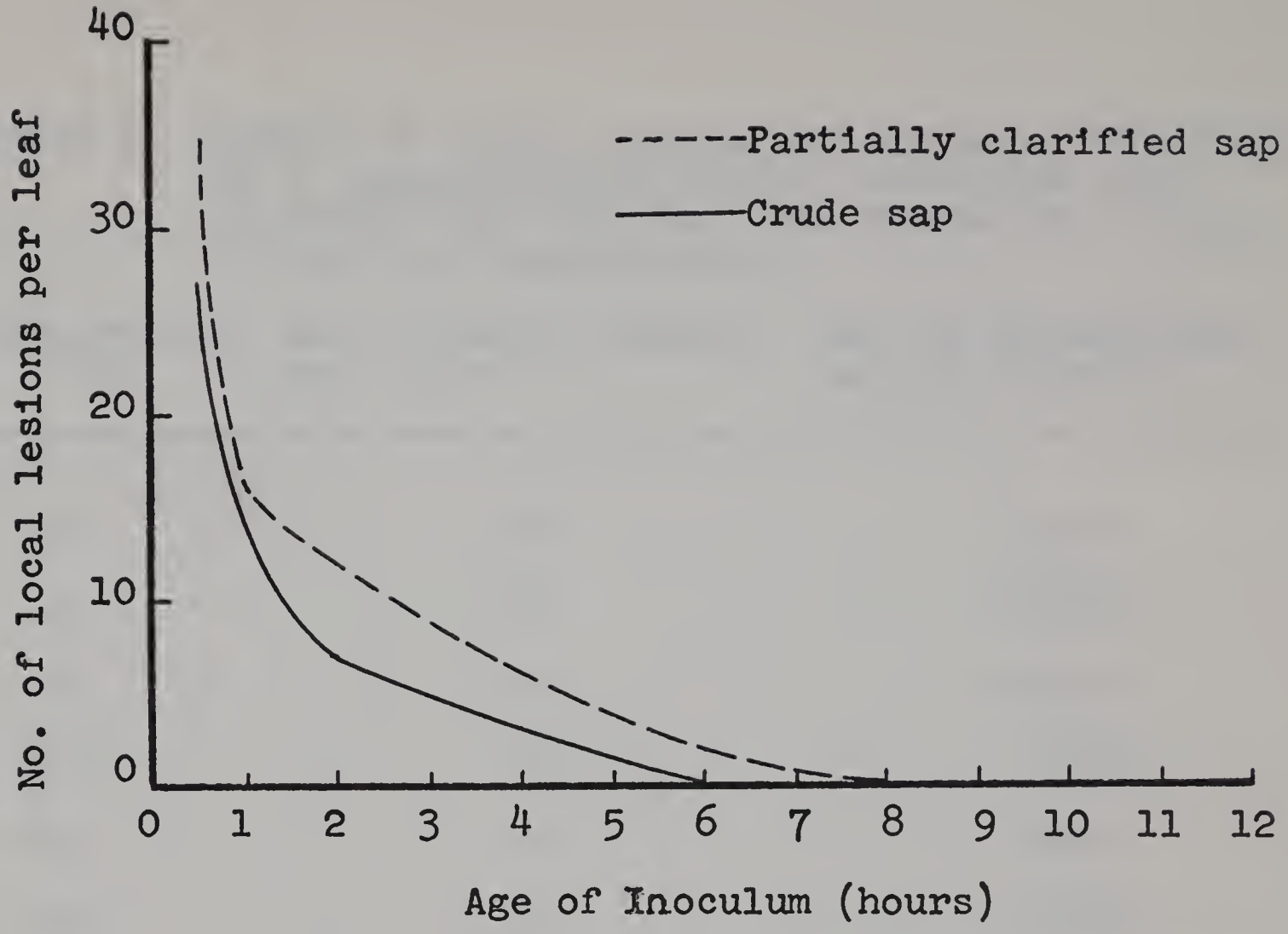
Dilutions	No. of local lesions per leaf**		No. of plants systemically infected	
	Crude sap	Partially clarified sap	Crude sap	Partially clarified sap
1: 5	29	31	25/25	25/25
1:10	21	24	17/25	25/25
$1:10^2$	14	19	7/25	25/25
$1:10^3$	3	7	2/25	14/25
$1:10^4$	0	2	0/25	2/25
$1:10^5$	0	0	0/25	0/25
$1:10^6$	0	0	0/25	0/25

*All data shown in this table are averages from 7 replicates.

**Average of the number of lesions of 6 inoculated leaves on each plant, from 7 replicates.

Fig. 1. Longevity *in vitro* of RRV in crude sap and in clarified sap kept at 24°C, as assayed by the number of local lesions per leaf on the inoculated *C. amaranticolor* plants

Fig. 2. Number of local lesions per leaf of *C. amaranticolor* of serial dilutions of RRV in crude sap and in partially clarified sap.


40
30
20
10
0
No. of local lesions per leaf
-----Partially clarified sap
——Crude sap
0 1 2 3 4 5 6 7 8 9 10 11 12
Age of Inoculum (hours)

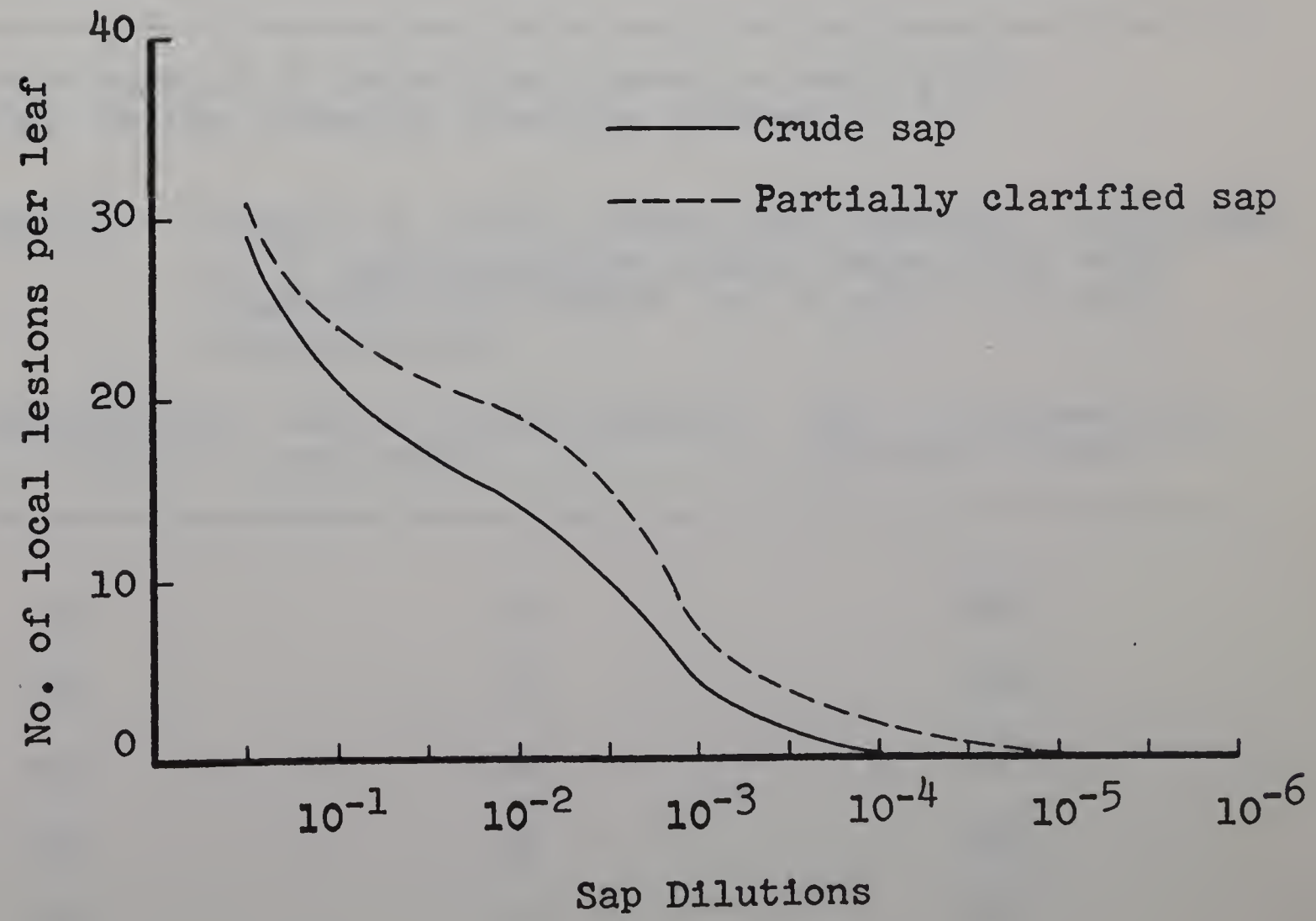

40
30
20
10
0
No. of local lesions per leaf
—— Crude sap
---- Partially clarified sap
10^{-1} 10^{-2} 10^{-3} 10^{-4} 10^{-5} 10^{-6}
Sap Dilutions

Table 3. Number of local lesions and systemic infections on C. amaranticolor plants inoculated with clarified sap that had been heated for 10 min at various temperatures.

Temperature (°C)	No. of local lesions per leaf*	No. of systemically infected plants
20	38	10/10
30	28	10/10
40	21	10/10
50	2	0/10
60	0	0/10
70	0	0/10
80	0	0/10
CK**	36	10/10

*Average of 6 inoculated leaves on each plant.
**Ck is the unheated inoculum control.

Table 4. Number of local lesions and systemic infections on C. amaranticolor plants inoculated with clarified sap heated for 10 min at various temperatures.

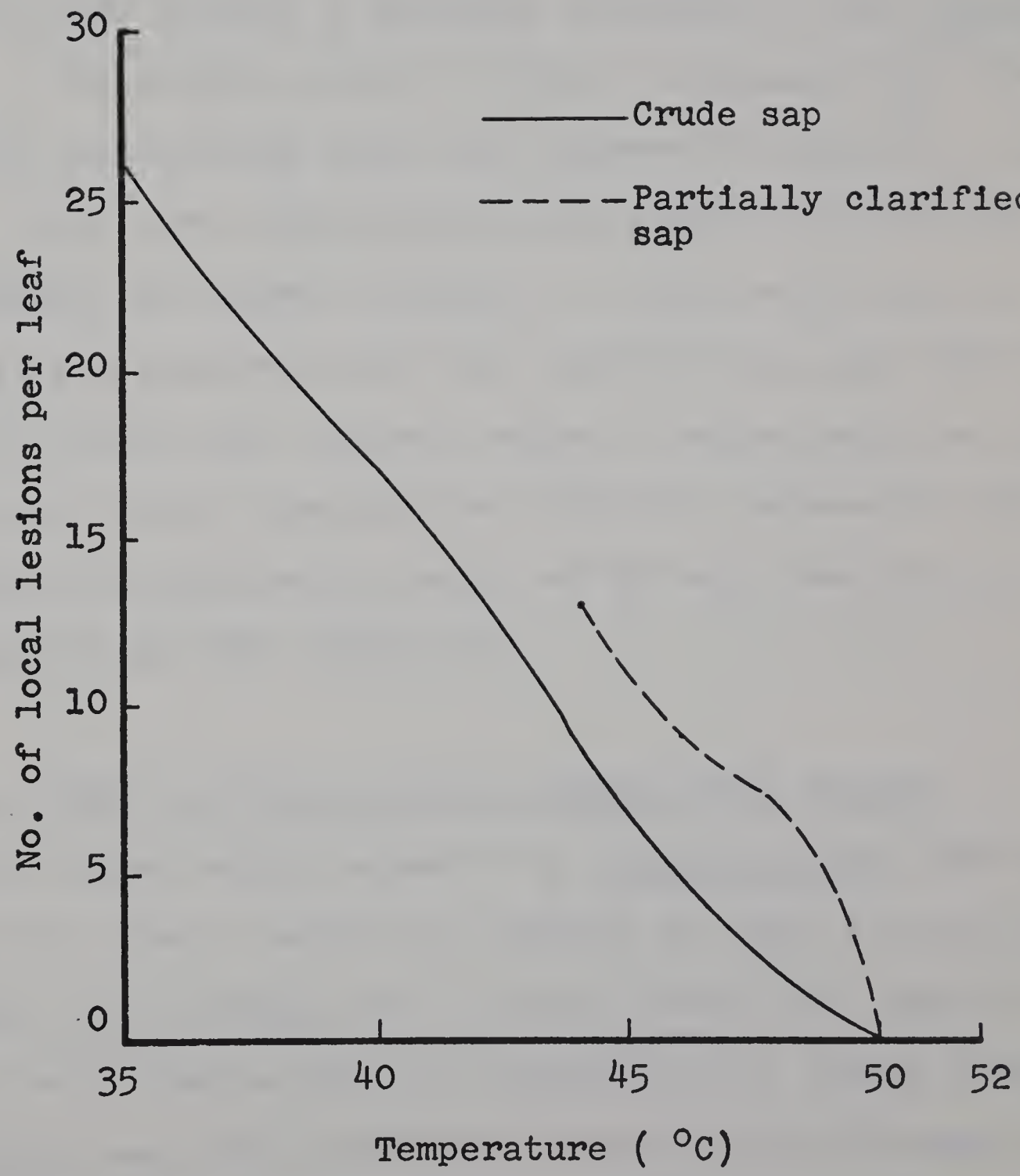
Temperature (°C)	No. of local lesions per leaf	No. of systemically infected plants
40	19	8/8
45	7	7/8
50	2	0/8
55	0	0/8
CK	37	8/8

Table 5. Number of local lesions and systemic infections on C. amaranticolor plants inoculated with either crude or partially clarified sap that had been heated for 10 min at various temperatures.

Temperature (°C)	No. of local lesions per leaf		No. of systemically infected plants	
	Crude sap	Partially clarified sap	Crude sap	Partially clarified sap
35	26	-	10/10	10/10
40	17	-	10/10	10/10
44	9	13	10/10	10/10
46	5	9	7/10	10/10
48	2	7	2/10	3/10
50	0	0	0/10	0/10
52	0	0	0/10	0/10
CK*	36	37	10/10	10/10

*CK is the unheated inoculum control.

Fig. 3. Number of local lesions on C. amaranticolor plants inoculated with either crude or partially clarified saps that had been heated for 10 min at various temperatures.


30
25
20
15
10
5
0
No. of local lesions per leaf
35
40
45
50
52
Temperature (°C)
Crude sap
Partially clarified sap

Crude sap was not infective when heated at 50°C for 10 min, but similarly treated clarified sap induced formation of lesions on the inoculated leaves (Tables 3 & 4), although they did not produce a systemic infection of the Chenopodium plant. The results shown in Table 3 indicated that the thermal inactivation point was between 40 and 60°C. Therefore, tests were subsequently made between 40 and 55°C, and the results are shown in Table 4. Since only trace of infectivity appeared after the clarified sap had been heated at 50°C for 10 min, another series of experiments was performed at shorter temperature intervals between 40 and 52°C. The thermal inactivation point of RRV was found to be between 48 and 50°C (Table 5).

Aging of RRV in frozen and in freeze-dried tissue:

In frozen whole leaves of C. amaranticolor, RRV was infective after 2 months of storage at -22°C in the freezer but not after storage for 4 months (Table 6). When the leaves were freeze-dried and ground into a coarse powder, the virus was still infective 9 months after storage at -22°C (Table 7). This experiment was repeated 3 times.

Table 6. Aging of RRV in frozen leaf tissue of C. amaranticolor as assayed on C. amaranticolor*

Time interval	No. of local lesions per leaf	No. of plants systemically infected
1 day	32	10/10
1 week	20	10/10
2 weeks	18	10/10
4 weeks	16	10/10
2 months	11	2/10
4 months	0	0/10
6 months	0	0/10

*All data shown in this table are averages from 3 replicates. The assay was made with partially clarified sap only.

Table 7. Aging of RRV in freeze-dried leaf tissue of C. amaranticolor as assayed on C. amaranticolor*

Time interval	No. of local lesions per leaf	No. of plants systemically infected
1 day	28	15/15
1 week	21	15/15
1 month	20	15/15
6 months	14	15/15
9 months	13	15/15

*All data shown in this table are averages from 4 replicates. The assay was made with partially clarified sap only.

PURIFICATION

Assay hosts:

Of the numerous herbaceous plant species inoculated with crude virus preparations, only species of Chenopodium produced good symptoms. Sunken or necrotic local lesions developed after 4 to 7 days on the inoculated leaves. The lesions were 0.5 to 1.0 mm in diameter and were scattered over the entire leaf surface (Fig. 4). Three to four weeks later, systemic symptoms developed on the non-inoculated new leaves formed at the tips, consisting of vein-clearing, yellowish-mottling and leaf distortion (Fig. 5). The local lesions were chlorotic at first but later became necrotic. Systemic symptoms disappeared when infected plants were kept at temperatures higher than 30°C for more than 12 hr. In addition to Chenopodium amaranticolor and C. quinoa, the bean varieties Bountiful stringless, Tenderbest, Kentucky Wonder and Pod Pencil Wax produced symptoms on inoculated cotyledons. Sunken local lesions, black to brown in color, developed about 3 days after inoculation on the inoculated cotyledons. The lesions were 0.1 to 0.5 mm in diameter, usually scattered near the margin of cotyledons. Lesions disappeared one week later. No systemic symptoms were observed.

Fig. 4. Local lesions on leaves of Chenopodium amaranticolor 7 days after inoculation with RRV.

Fig. 5. Systemic infection on leaves of Chenopodium amaranticolor 2 weeks after inoculation with RRV.

Infectivity test:

The sample leaves were ground, the sap was clarified by Mg-bentonite, and the homogenate was centrifuged at 10,000 rpm in a Sorvall RC2-B, SM-24 rotor for 15 min to get rid of the coarse plant material. The supernatant (I) was used to inoculate the assay hosts to make sure that the virus was still present in an infectious form. This supernatant (I) was found infective and produced an average of 31 local lesions per leaf while the pellet was not infective. Supernatant (I) was then centrifuged at 30,000 rpm in a Spinco L2-65B, No. 65 rotor for 1 hr. The resulting supernatant (II) and the resuspended pellet (II) were each inoculated on the assay hosts. Supernatant (II) caused no infection on assay hosts while the resuspended pellet (II) caused an average of 116 local lesions per leaf on C. amaranticolor followed by systemic infection of all (20) plants inoculated.

Concentration of virus by ultracentrifugation:

According to the infectivity assay experiments, after high speed ultracentrifugation, the pellet resuspension produced 116 local lesions per leaf on the assay hosts; and it continued to be infective even at a dilution of $1:10^6$, at which it caused about 2 local lesions per inocu-

lated leaf. In contrast, partially clarified sap caused only an average of 31 local lesions per inoculated leaf and crude sap caused on the average 27 local lesions per leaf. Ultracentrifugation, then, did increase the virus concentration in the suspension. The infectivity assay tests were again made on Chenopodium amaranticolor and on Phaseolus vulgaris vars. Bountiful stringless and Kentucky Wonder (Table 8 and Fig. 6).

Purification by density gradient centrifugation:

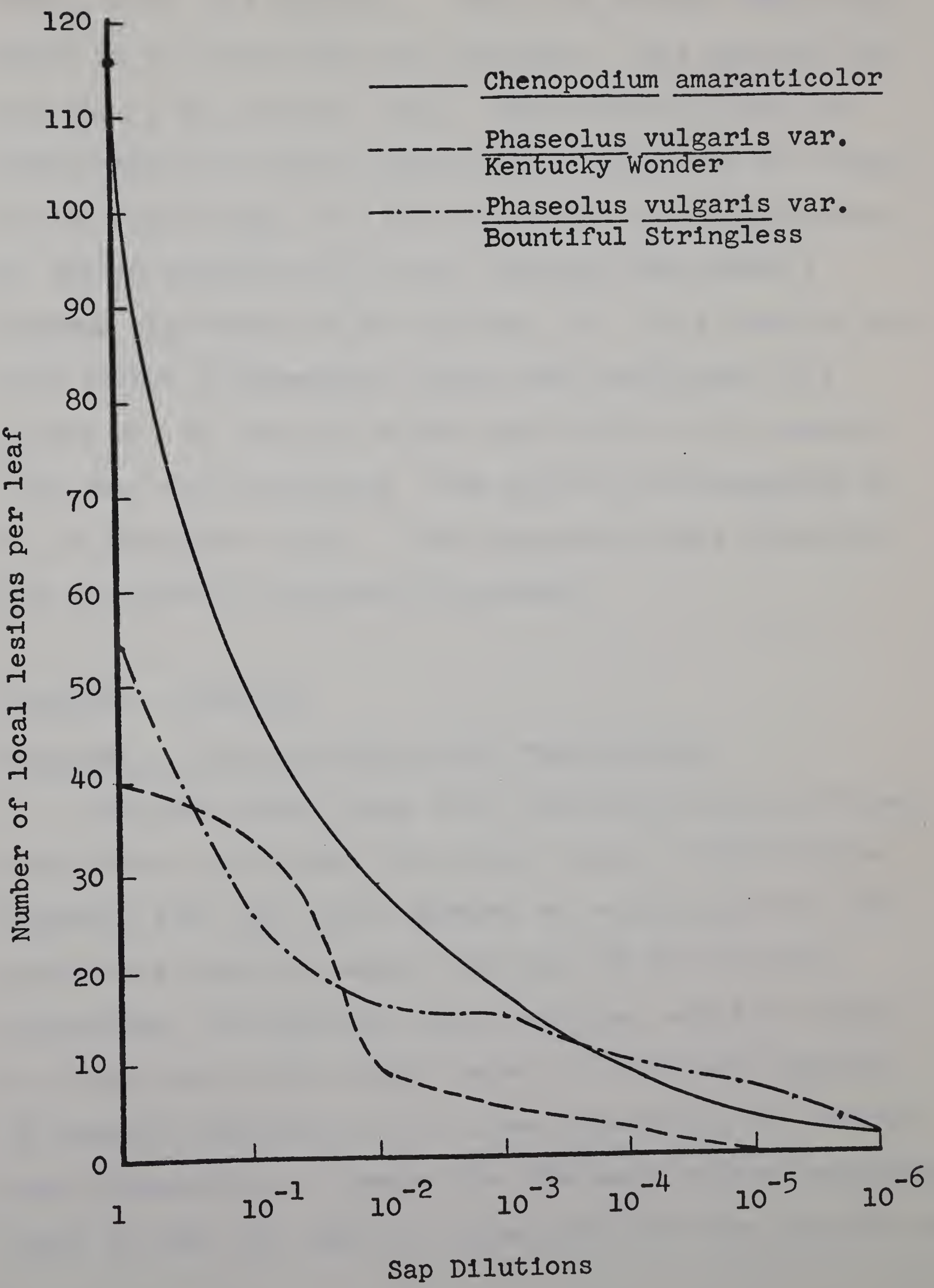
For further purification of the virus, density gradient centrifugation was applied. No virus zones were seen in the gradient tubes in initial experiments, but localization of infectivity in definite regions of the tubes suggested that density gradient centrifugation does concentrate this virus in an infective form. One ml of purified virus suspension (obtained from 25g tissue of systemically infected C. amaranticolor leaves concentrated into 2 ml virus suspension in buffer) was layered on the top of rate-zonal density gradient tubes. After 4 hr of centrifugation at 28,000 rpm in a Spinco SW 40 rotor, there was a wide, faint band at about 12 mm below the meniscus. The width of the band was about 9 to 10 mm. This band was also found in the gradient tube with the control preparation

Table 8. Local lesion assay of a purified RRV.

Virus dilutions in buffer	Number of local lesions per leaf*		
	Chenopodium amaranticolor	Phaseolus vulgaris var. Kentucky Wonder	Phaseolus vulgaris var. Bountiful Stringless
1:1	116	40	54
1:10	49	34	26
$1:10^2$	29	9	16
$1:10^3$	17	5	15
$1:10^4$	9	3	10
$1:10^5$	4	0	7
$1:10^6$	2	0	2

*Average number of lesions from 6 inoculated leaves on each of 15 C. amaranticolor plants and of 2 inoculated cotyledons on each of 10 bean plants.

Fig. 6. Local lesion assay of different dilutions of purified Russet Ring Virus preparation on three assay hosts.


120
110
100
90
80
70
60
50
40
30
20
10
0
Number of local lesions per leaf
1
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
Sap Dilutions
Chenopodium amaranticolor
Phaseolus vulgaris var. Kentucky Wonder
Phaseolus vulgaris var. Bountiful Stringless

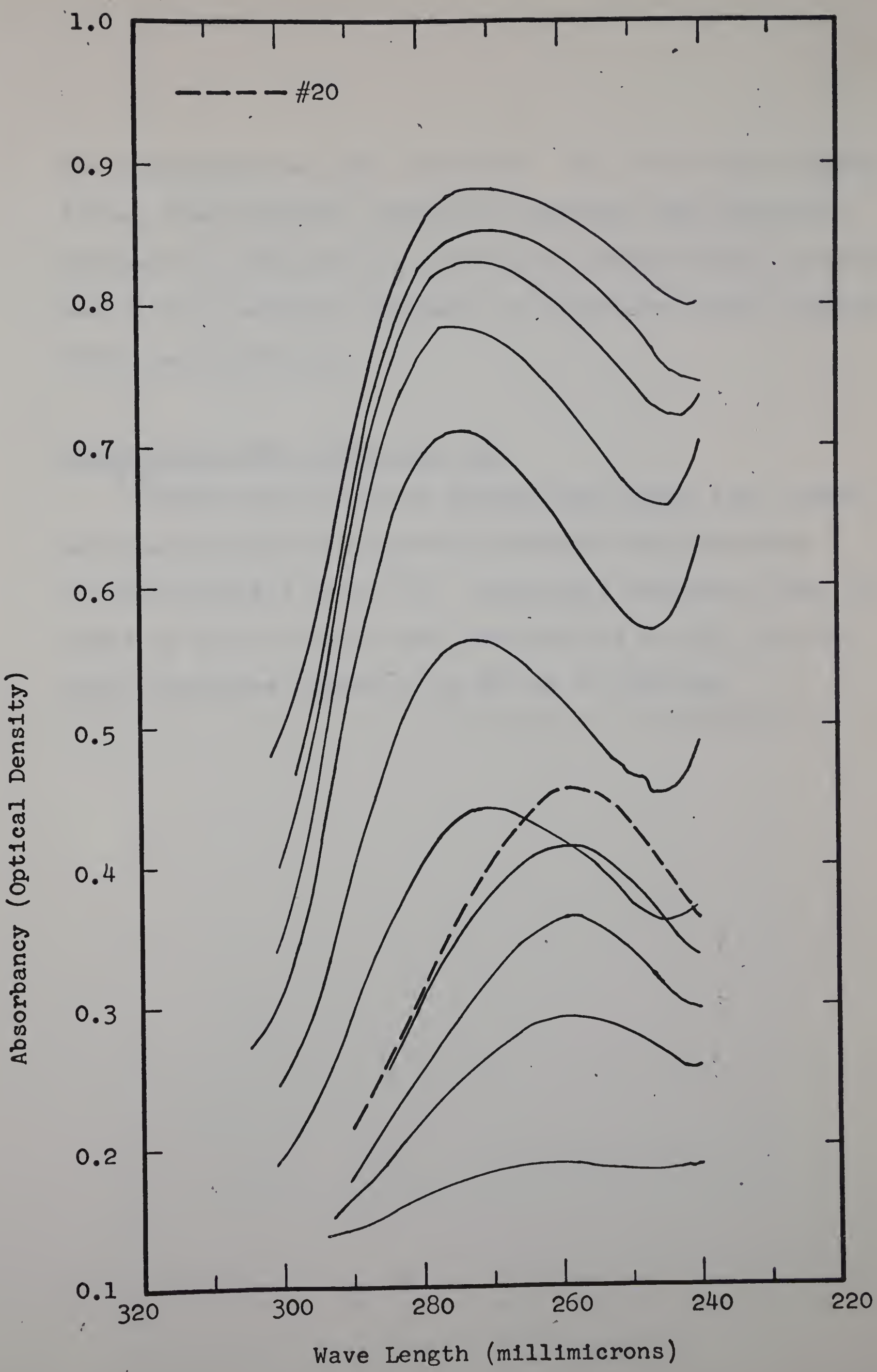
from health leaf material. There was another clear band about 22 to 23 mm below the meniscus. This band was not present in the control tubes. Each gradient tube was fractionated and each fraction was examined at the ultraviolet light range in a Spectronic 505 spectrophotometer. Of the 28 fractions per tube, fraction #20 showed a maximum absorption at 260 mμ (Fig. 7). This fraction was then washed in phosphate buffer and centrifuged in a Spinco No. 65 rotor at 30,000 rpm for 60 min to precipitate the virus particles. The pellet was resuspended in 0.1 M phosphate buffer. This preparation was infectious and was used for electron microscopy.

ELECTRON MICROSCOPY

Preparation from purified virus resuspension:

Purified russet ring virus preparation from different experiments were mixed with equal volume of 1% phosphotungstic acid (pH 7.05), sprayed on carbon grids and the grids were examined under a Phillips EM 200 electron microscope. No definite virus particles could be found, but there were often large numbers of ribosomes present. In several experiments, the virus preparation was treated with ribonuclease to remove the ribosomes before examination under the EM, but again no virus particles were observed and

Fig. 7. Ultraviolet absorption curves of fractions obtained from one density-gradient centrifugation tube.


#20
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Absorbancy (Optical Density)
320
300
280
260
240
220
Wave Length (millimicrons)

the preparation was not infective. In a few virus preparations, some flexuous, elongated particles were observed. Although it could not be ascertained whether these particles were virus, membrane fragments or artifacts, these preparations were infective.

Preparation from crude leaf sap:

Systemically-infected Chenopodium quinoa leaf tissue was homogenized and negatively stained with potassium phosphotungstate at pH 7.0. Virus-like particles (Fig. 8) could be found in most such preparations and the size of these particles seemed to be 920 nm x 15-20 nm.

Fig. 8. Electron micrograph of a crude sap preparation of RRV from systemically-infected Chenopodium quinoa showing a filamentous virus particle (x 54,000)

V: Virus particle
R: Ribosomes


V
R
V
500 nm

DISCUSSION

RRV was mechanically transmitted from apple leaves and petals to Chenopodium and then retransmitted from Chenopodium to Chenopodium and to bean. Several environmental factors, especially light and temperature, were important for sucessful transmission of the virus. In summer months, the greenhouse had to be shaded to lower the temperature. In winter months, artificial lighting was used to keep the light intensity at a minimum of 500 ft-c. High temperatures affected the expression of systemic symptoms on the non-inoculated tip leaves. When the temperature rose above 30°C, it masked all systemic symptoms. The photoperiod was also important and had to be adjusted to keep host plants from flowering in order to allow development of systemic symptoms and to provide enough leaf material for inoculum and purification. Chenopodium is a short-day plant which flowers when it receives about 8 hr of light, but photoperiods of 16 hr or more inhibit flowering (Schwale, 1959). The age of host plants also played an important role in transmission. When the plants were too young, the inoculation often killed the whole plant; while when they were quite old, no symptoms would appear following inoculation. Usually, the Chenopodium plants were inoculated when they had 6 to

8 expanded leaves.

Pre-inoculation treatment was necessary for both Chenopodium and bean plants. Plants to be inoculated were kept in darkness for 48 hr before inoculation. Untreated plants did not show symptoms when inoculated by the same method. Mg-bentonite, used to clarify the virus preparation, could affect the infectivity of the preparation considerably. Too much bentonite, generally, destroyed the infectivity of the preparation.

In studying the aging of RRV in vitro at room temperature, it was noted that clarified sap was infective for only 2 hr longer than was the crude sap. The dilution end point of RRV was tested both in crude and clarified saps. This experiment was repeated 7 times because in the initial experiments the results were quite variable. Finally, it was determined that the variation was due to masking of the symptoms on the inoculated plants due to the high temperature (37.8°C or more) in the greenhouse during the summer. When the experiments were carried out at lower temperatures, the results were normal and repeatable. The results indicated that the dilution end point of RRV is between $1:10^3$ and $1:10^4$ (crude sap). The study of the thermal inactivation point of RRV was rather complicated because of the various temperatures in the greenhouse during the summer. When all

virus. This second diffuse band was eluted from the sucrose density-gradient tube as fraction #20. This fraction exhibited a good UV absorption curve in the Spectrophotometer. After washing this fraction twice with phosphate buffer, it was concentrated into 0.1 ml and was prepared for electron microscopy. Unfortunately, no virus particles could be found in that fraction when it was examined with the EM. The first wash, which took place in a Spinco No. 65 rotor at 60,000 rpm for 1 hr, might have caused the breakdown of the virus particles, but there is no proof for that. When the preparation was incubated for 1 hr at 37°C with a ribonuclease to remove the ribosomes, removal of the ribosomes still did not reveal any virus particles. Virus particles, however, were found in the crude leaf sap preparations. In these RRV appeared as a filamentous particle with a length about 920 nm and width of 15-20 nm.

The virus studied here was very similar, in some respects, to the CLSV purified by Saksena and Mink (1969). Table 9 shows a comparison of some properties of RRV and CLSV. The similarity of the properties of RRV and CLSV indicates that the two viruses could be closely related or they may even be strains of the same virus. However, the symptoms caused by these viruses are quite different. RRV affects leaves and fruits of McIntosh apple (Agrios, 1965)

tests were made in the growth chamber which was kept at 24°C during the day and 15.6°C at night, it was shown that the thermal inactivation point of RRV was between 48°C and 50°C. The longest storage period of freeze-dried, virus-infected leaf tissue was tested was 9 months, which was thought to be sufficiently long for keeping an infected sample for experimental purposes. The virus was not inactivated during those 9 months, but it was not determined how much longer RRV could remain infective in the freeze-dried leaf tissue.

The procedures used for purification of RRV in this study were similar to those previously used by Saksena and Mink(1969) for purifying apple chlorotic leafspot virus (CLSV), with some necessary modifications. The speed and times of centrifugation of RRV were different from those used for CLSV. Although the purified virus suspension showed a very good UV absorption curve at 260 mμ when it was examined in the Spectrophotometer, and was highly infective, only two very diffuse bands could be detected in the sucrose density-gradient tubes. These bands did not stand out sufficiently for satisfactory photography. The top band appeared to consist of normal plant ribosomes, but the second band from the meniscus was absent in the control tubes, suggesting that it probable contained the

while CLSV does not. CLSV causes chlorotic spots and sometimes die back on Russian Clone apple seedlings and on Spy 227 apple (Lister et al., 1964) while RRV does not. The virus particles of RRV and CLSV are similar in shape but not in size. CLSV has filamentous particles with 500 to 700 nm in length (Lister et al., 1965; Saksena and Mink, 1969) and the RRV particles are also filamentous but about 920 nm or more in length.

The information obtained by this study should help in identifying the virus and should provide basic information that could be used in further investigation of the virus regarding its nature and its control.

Further work, including a direct comparison of the properties of RRV and CLSV, especially through production and cross-reaction of specific antisera produced against each of the two viruses, is needed to establish the identity of RRV and its relationship to CLSV.

Table 9. Comparison of some properties of apple russet ring virus (RRV) and apple chlorotic leafspot virus (CLSV).

Properties	RRV	CLSV*
Dilution end point	Between 1:1,000 and 1:10,000	Between 1:1,000 and 1:5,000
Thermal inactivation point	Between 48 and 50°C	Between 50 and 55°C
Aging *in vitro*	6 hours	7 hours
Length of virus particles	920 nm**	500-700 nm

*The data for CLSV are from Lister et al.,(1965) and Saksena and Mink (1969).
**Average of 8 virus-like particles found under the EM.

SUMMARY

Russet ring virus (RRV) of apple infects McIntosh apples in Massachusetts and is mechanically transmitted to herbaceous hosts, Chenopodium amaranticolor and C. quinoa.

Certain properties of this virus were studied, and attempts were made to purify the virus and view it under the electron microscope. Retransmission of RRV from C. amaranticolor to C. amaranticolor produced local lesions followed by systemic infection. Best infection results are obtained in a growth chamber or greenhouse kept at 24°C and at light intensities between 500 and 800 ft-c. Chenopodium plants should be inoculated when they have 6 to 8 expanded leaves and are about 5 inches tall. A 48-hr pre-inoculation darkness treatment of hosts is necessary.

The inoculum was prepared by adding 4 parts of 0.01 M phosphate buffer containing 0.02 M sodium diethyldithiocarbamate and 0.02 M cysteine hydrochloride to one part of infected leaf material. Mg-bentonite suspension was added at 2 ml/5g tissue. The mixture was centrifuged at 19,000 rpm for 15 min in a Sorvall RC-2, SM-24 rotor. The supernatant was used as inoculum.

The dilution end point of RRV in crude sap is between $1:10^3$ and $1:10^4$, and in bentonite-clarified sap between

$1:10^4$ and $1:10^5$. Only a trace of infectivity remained in crude sap heated at 48°C for 10 min and there was no infectivity after 10 min at 50°C. Crude and clarified saps stored at 24°C lost all infectivity in approximately 6 hr. RRV was still infective after 2 months storage in frozen leaf tissue and after 9 months in freeze-dried leaf tissue powder.

The purification of RRV was achieved by low-high speed centrifugation at 10,000 rpm for 15 min in Sorvall RC-2, SM-34 rotor, and at 30,000 rpm for 60 min in a Spinco 65 rotor, respectively. Further purification was carried out by sucrose density-gradient centrifugation. The concentrated virus suspension was used for electron microscopy. The RRV particles was found to be filamentous in shape and about 920 nm long by 15-20 nm in diameter.

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